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Transcriptional behavior of EUL-related rice lectins toward important abiotic and biotic stresses

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SUMMARY

The rice genome encodes several genes for putative carbohydrate-binding proteins belonging to the family of *Euonymus* related lectins (EULs). This lectin family was discovered recently and evidence shows that the expression of these proteins is subject to multiple environmental stresses. In this study, quantitative reverse transcription PCR (qRT-PCR) was conducted on rice seedlings exposed to various abiotic (150 mM NaCl, 100 mM mannitol, and 100 μM abscisic acid (ABA)) and biotic (*Xanthomonas oryzae* pv. *oryzae* and *Magnaporthe oryzae*) stresses to compare the transcriptional behavior of the EULs and a known stress related lectin Orysata belonging to the family of jacalin-related lectins. All EUL transcripts were strongly up-regulated after ABA and NaCl treatments in the roots whereas the overall expression level was generally lower and more variable in the shoots. Moreover, all abiotic stresses induced Orysata in both tissues except for mannitol treatment which failed to show an effect in the roots. Orysata also strongly accumulated after *X. oryzae* pv. *oryzae* infection, as were various D-type EUL lectins. In contrast, some of the EUL proteins, including OrysaEULS3, OrysaEULD1A and OrysaEULD2, as well as Orysata were significantly down-regulated upon *M. oryzae* attack, suggesting fungal manipulation of these genes. Collectively, our results clearly show that rice expresses multiple carbohydrate-binding proteins in response to a wide variety of abiotic and biotic stress conditions. We hypothesize that the *Euonymus* related proteins fulfill a prominent role in sensing and responding to multiple environmental cues.

Introduction

Plants are sessile organisms, and therefore have evolved a set of sophisticated mechanisms to adapt to the daily and seasonably fluctuating environment. Perception of environmental cues is known to trigger a variety of plant responses, including the expression of large suites of stress-responsive proteins such as lectins. Lectins are proteins with the ability to specifically and reversibly recognize and bind different carbohydrate structures that are either present inside the plant tissue or occurring on other organisms (Peumans and Van Damme, 1995). About a decade ago, it was shown that the expression of particular lectins is stress related (Van Damme et al., 2004), in that lectin expression is induced after salt stress (Zhang et al., 2000), pathogen infection (Qin et al., 2003), jasmonic acid treatment (Lannoo et al., 2007) and insect herbivory (Vandenborre et al., 2009). Since most of the inducible lectins are localized in the nucleus and/or the cytoplasm, they are also referred to as nucleocytoplasmic lectins. The hypothesis was put forward that these inducible lectins play a role in the stress physiology of the plant (Van Damme et al., 2008).

One family of inducible lectins that is ubiquitous in plants comprises those proteins related to the *Euonymus europaeus* lectin (EUL) (Fouquaert et al., 2009). Though the EUL domain is quite well conserved, the carbohydrate-binding domain is promiscuous in that it shows interaction with different carbohydrate structures depending on the species and the type of protein under study. Based on the protein domain architecture two types of protein can be distinguished. Type S proteins contain a single EUL domain preceded by a variable N-terminal sequence, whereas type D proteins are composed of two tandem arrayed EUL domains separated by a linker sequence and preceded by an N-terminal sequence (Fouquaert and Van Damme, 2012).

In rice, at least four different types of *Euonymus*-related proteins have been identified (Fouquaert et al., 2009; Fouquaert and Van Damme, 2012). Two proteins are of the S type, referred to

Abbreviations: ABA, abscisic acid; EUL, *Euonymus europaeus* lectin.

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as OrysaEULS2 for type S2 and OrysaEULS3 for type S3, respectively. In addition several D type proteins with two EUL domains were identified. The size of the linker and N-terminal domains differs in case of the D1 and D2 types. Two almost identical proteins referred to as OrysaEULD1A and OrysaEULD1B belong to the D1 type, whereas one protein was classified as a D2 type, namely OrysaEULD2 (Fouquaert et al., 2009; Al Atalah et al., 2013).

Rice (*Oryza sativa L.*) is one of the most important cereal crops and provides nearly 20% of the daily calories for more than 3.5 billion people worldwide (Khush, 2013). As for many other crops, rice yields are severely compromised by a combination of biotic and abiotic stresses. Among the abiotic stresses, salinity and drought have received most attention. Salinity adversely affects both the quantity and the quality of the crop yield (Blumwald and Grover, 2006; Hauser et al., 2011). Drought is the most important limiting factor for crop production in many regions of the world (Passioura, 2007; Witcombe et al., 2008). Among the biotic stresses, bacterial leaf blight (*Xanthomonas oryzae pv. oryzae*) and rice blast (*Magnaporthe oryzae*) are the most important constraints on high rice productivity (Talbot, 2003; Sana et al., 2010; De Vleesschauwer et al., 2013). Considering that combined abiotic and biotic stresses take an estimated 30–60% bite out of potential crop yield, deciphering the molecular mechanisms by which plants process and integrate diverse stress signals is of fundamental importance (Mittler and Blumwald, 2010; Seo et al., 2011).

In the present study, we used quantitative reverse transcription-PCR (qRT-PCR) to analyze the expression profile of all EUL proteins from rice under different abiotic and biotic stress conditions. Quantitative RT-PCR has become a pioneer technology to probe gene expression because of its rapidity, accuracy and sensitivity (Gingeras et al., 2005; Nolan et al., 2006; Van Guilder et al., 2008). In addition, qRT-PCR is considered the most appropriate method to confirm or confute data generated by large scale microarrays (Wang et al., 2006). Our findings can contribute in deciphering the role and biological significance of inducible EUL lectins in the plant stress signaling network. Furthermore, we have proven that EUL lectins are stress-related proteins which gives the possibility of exploiting these inducible lectins to increase the tolerance of rice plants against the environmental cues.

Materials and methods

Plant material

Oryza sativa cv *Nipponbare* seeds were obtained from the National Rice Research Center (Genetic stock Oryza collection, Stuttgart, USA). To initiate *in vitro* cultures, seeds were de-husked and surface sterilized in 70% (v/v) ethanol for 5 min and subsequently in 5% (v/v) NaOCl for 30 min. Afterwards the seeds were extensively washed with sterile water.

Abiotic stress assays

Ten seeds were sown in a jar (9 cm diameter) containing 100 ml solid Murashige and Skoog (1962) medium (MS) (4.3 g/l MS micro- and macronutrients containing vitamins (Duchefa, Haarlem, The Netherlands), 30 g/l sucrose, pH 5.7 (adjusted with 0.5 M NaOH), and 8 g/l plant agar (Duchefa)). The jars were kept at 28 °C, 16 h light/8 h dark cycle.

After 10 days, 5 seedlings were transferred to a jar with 100 ml liquid MS containing the desired stress factor (150 mM NaCl, 100 mM mannitol or 100 μM abscisic acid (ABA)). In addition, five seedlings were transferred to a jar containing only MS and used as a control. Two jars (10 seedlings in total) were used for each treatment. The stress treatment was applied for 6 h under the conditions mentioned above. Afterwards, the shoots and the roots were separated and immediately frozen at −80 °C.

Biotic stress assays

Sterilized rice seeds were incubated on wet sterile filter paper for five days at 28 °C. In case of *Xanthomonas oryzae pv. oryzae* bioassays, germinated seedlings were transferred to sterilized vermiculite supplemented with half strength Hoagland solution (Xu et al., 2013). Two weeks later, the plants were transferred to plastic containers containing modified Hoagland solution (Xu et al., 2013) and grown for another three weeks under growth chamber conditions (28 °C, relative humidity: 60%, 12/12 light regime). Seedlings used for *Magnaporthe oryzae* inoculations were grown in perforated plastic trays (23 by 16 by 6 cm) filled with commercial potting soil (Structural; Snebbout, Kaprijke, Belgium) that had been autoclaved twice on alternate days for 21 min.

X. oryzae pv. oryzae assay

X. oryzae pv. oryzae strain PXO99 (Philippine race 6) (Song et al., 1995) was routinely cultured on Sucrose Peptone Agar (SPA) medium at 28 °C. For inoculation, a single colony was transferred to liquid SP medium and grown for 48 h at 28 °C. Six week old plants were inoculated by clipping the fifth and sixth stage leaves with scissors and dipping them in a solution of *X. oryzae pv. oryzae* cells in water (1×10^9 CFU ml^{-1}). Inoculated plants were kept in a dew chamber (≥92% relative humidity; 28 °C) for 24 h and thereafter transferred to growth chamber conditions for disease development. Leaf samples of mock and pathogen-inoculated plants were collected 2, 4 and 8 days post-inoculation (dpi) and frozen at −80 °C until use. Successful plant infection was confirmed by the appearance of long (>10 cm), water-soaked blight lesions at 14 dpi (Xu et al., 2013).

M. oryzae assay

Inoculations with *M. oryzae* were performed exactly as described in De Vleesschauwer et al. (2009). Briefly, leaves of 4-week-old plants (5-leaf stage) were evenly sprayed with a *M. oryzae* spore suspension containing 5×10^4 conidia ml^{-1}. Inoculated plants were incubated at high relative humidity (≥92% relative humidity; 28 °C) for 24 h and, thereafter, returned to growth chamber conditions for disease development. Six days post-inoculation, inoculated leaves were found to display many sporulating blast lesions, confirming the effectiveness of the pathogen inoculation. Leaf samples of mock and pathogen-inoculated plants were collected at 1 and 2 dpi, and frozen at −80 °C until further use.

RNA extraction and cDNA synthesis

Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN, Valencia CA, USA) following the manufacturer's instructions. After DNase I treatment (ThermoScientific, Erembodegem, Belgium), cDNA was synthesized using the M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). Briefly, in a total volume of 26 μl, 2 μg of DNase I treated RNA were mixed with 2 μl of 2 μM oligo-dT and 2 μl of 10 mM dNTP. After incubation for 5 min at 65 °C all samples were cooled down immediately. Subsequently the samples were mixed with 8 μl of the 5× first strand buffer and 4 μl of 0.1 M dithiothreitol. After 2 min incubation at 37 °C, 2 μl M-MLV reverse transcriptase (200 U/μl) was added. After incubation for 50 min at 37 °C all samples were transferred to 75 °C for 15 min to stop the reaction. Finally, all samples were diluted to a concentration of 20 ng/μl. To check the quality of the cDNA, a standard RT-PCR